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Protein processing in *Plasmodium falciparum?*

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ABSTRACT

The genomes from the organisms of the *Plasmodium* genus, the causative agents of human and animal malaria, are characterized by an extreme high A+T content and an associated abundant low complexity inserts within their proteins. The enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (G6PD-6PGL) found in *Plasmodium* species has unique structural and bifunctional characteristics. Here, we report the expression analysis of *P. faciparum* G6PD-6PGL along the intraerythrocytic cycle by immunological analysis with antibodies raised against its N- and C- terminal domains. The pattern modification of band sizes at the different stages of parasite development suggest intracellular protein processing involving the cleavage of the native bifunctional form to produce two main fragments. *In vitro* RNA-mediated PfG6PD-6PGL gene silencing, studied along short-term parasite development also revealed the apparent intracellular protein modification dependent on the parasite stage. Fragment sizes were consistent with separating both catalytic functions of the enzyme. The proteolytic machinery underlying this specific PfG6PD-6PGL proccesing is still unknown in *P. falciparum* but suggests the existence of distinctive mechanisms in the parasite to deal with unique protein structures of essential function resulting from its genome evolution.

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Key words: Malaria.—Protein processing.—Glucose-6-phosphate dehydrogenase.—Gene silencing.—dsRNA.

RESUMEN

¿Procesamiento de proteínas en *Plasmodium falciparum?*

Los genomas de los organismos del género *Plasmodium,* agente causante de la malaria humana y animal, se caracterizan por un alto contenido en A+T e inserciones de baja complejidad en sus proteínas. La enzima glucosa-6-fosfato deshidrogenasa-6-fosfogluconolactonasa (G6PD-6PGL) de las especies de *Plasmodium* posee unas características estructurales y bifuncionales únicas. En el presente trabajo analizamos la expresión de la G6PD-6PGL de *P. faciparum* a lo largo del ciclo intraeritrocítico mediante análisis inmunológico con anticuerpos frente a sus dominios N- y C- terminal. La modificación del tamaño del patrón de bandas en los diferentes estadios del desarrollo del parásito sugiere un procesamiento intracelular de la proteína que implicaría que la forma nativa bifuncional genera dos fragmentos principales. El silenciamiento *in vitro* del gen PfG6PD-6PGL, mediante ARN de interferencia, durante el desarrollo a corto plazo del parásito, también reveló la aparente modificación intracelular de la proteína dependiente del estadio de su ciclo vital. El tamaño de los fragmentos fue consistente con la separación de las dos funciones catalíticas de la enzima. Aunque en *P. falciparum* no se ha identificado la maquinaria proteolítica de este procesamiento específico de PfG6PD-6PGL, nuestros resultados sugieren la existencia de mecanismos especializados para el procesamiento intracelular de este tipo de proteínas de estructura única y de función esencial, y que han podido aparecer como consecuencia de la particular evolución de su genoma.

Palabras clave: Malaria.—Glucosa-6-fosfato deshidrogenasa.—Silenciamiento de genes.—ARN de interferencia.

INTRODUCTION

Plasmodium falciparum is the causative agent of the most severe form of human malaria being responsible for the death of more than 1 million people a year (1). The efforts to control the illness have been focused on the chemotherapy, mosquito control and in the development of vaccines. Nowadays, the lack of an effective vaccine and the increasing parasite resistance to available drugs suggest the necessity to identify new targets that allow the development of new drugs and vaccines. Completion of *Plasmodium*

genome sequences (2-4) has provided a vast amount of molecular information. This, together with the transcriptome (5, 6) and proteomic analysis along the parasitic developmental stages (7) pursue a deep understanding of the peculiar parasite biology in the context of exploring new therapeutic and immunization strategies. Since 1993, different technologies of *P. falciparum* genetic manipulation have been developed as gene disruption by homologous recombination (8-13), antisense RNA (14, 15) and more recently, RNA interference that has been successfully applied to understand the *in vivo* functions of *P. falciparum* genes (16-18).

Genome comparison from lower eukaryotes have shown that *Plasmodium* proteins are notably longer in size than their respective orthologous genes. This is due to the extraordinary attribute of *Plasmodium* proteins that are hugely enriched in stretches biased toward 1-3 residues (mainly Asn, Lys, and Ile) due to the high A+T content (about 80% average). Due to this particular composition these stretches acquire low entropy or complexity (19). These regions are embedded into highly conserved domains that form globular structures with larger variety of residue composition with high entropy and complexity (19). Although many eukaryotes have also low complexity areas in nuclear proteins, transcription factors, and some cytoskeletal proteins, *Plasmodium* species display low complexity stretches in unique genes, not observed before in genomes from other organisms. In addition, these unusual regions even penetrate in independent functional domains with unpredictably length between 10 and 100 residues (20).

P. falciparum G6PD-6PGL is a bifunctional enzyme exclusive to *Plasmodium* species (21) that probably arose from the fusion of two genes in a common ancestor (22). The deduced protein has a subunit molecular mass of 107 kDa, in agreement with the tetramer molecular weight calculated by size exclusion chromatography (23). Its C-terminal half (residues 311-911) is clearly homologous to other described G6PDs (with glucose 6-phosphate dehydrogenase activity) though sequence similarity is interrupted by a 62 amino-acid stretch with no similarity found to date. It has been nevertheless experimentally shown that this 62 amino acid insertion is essential for the activity of the bifunctional enzyme (24). In contrast, the 310 amino-acid protein sequence of the amino

terminal region clearly differs from most eukaryotic and prokaryotic G6PDs, and shows 6-phosphogluconolactonase activity; thus G6PD-6PGL catalyses the first two stages of the pentose phosphate pathway (21). The occurrence of large insertion sequences that differ with respect to their homologous proteins in other species has been often observed in many gene products of *P. falciparum* and other *Plasmodium* species, but their structural functions and origins are unknown (24, 25). We have been interested to know whether the low complexity invasions of globular domains could have a role in regulating protein turnover along the parasitic cycle, and therefore, in the present paper, the unique bifunctional *P. falciparum* G6PD-6PGL protein was explored as a model gene to study dynamics of its protein processing along the intraerythrocytic cycle and under gene silencing conditions.

EXPERIMENTAL PROCEDURES

Parasite cultures and electroporation

P. falciparum strain 3D7 was grown and double synchronised using standard procedures (26,27). Parasites (ring stage 8-10% parasitaemia) were transfected by electroporation with 40 µg of dsRNA as described (8). The parasites transfected with dsRNA-G6PD or dsRNA-Rab5a were kept for 24 h in 75 cm^2 flasks. The growth and development of each transfection was monitored by Giemsa staining blood films.

dsRNA design

A 21 basepair dsRNA (sense: UACAUCAUGCACCAACGAAdTdT; antisense: UUCGUUGGUGCAUGAUGUAdTdT) was designed for the target sequence (UACAUCAUGCACCAACGAA) of the G6PD-6PGL gene, following Dharmacon siDESIGN Center criteria (http://design.dharmacon.com/). In addition, a dsRNA corresponding to the PfRab5a gene (GenBank™ accession number AE001399) (target sequence: UAUGCAAGUAUUGUCCCAC; sense: UAUGCAAGUAUUGUCCCACdTdT; antisense: GUGGGACAAUACUUGCAUAdTdT)

was also designed to use as control. All dsRNAs were obtained from Dharmacon Research (Lafayette, CO, USA) in annealed and lyophilised form and were suspended in RNase-Dnase-free water before use.

Immunodetection

Antibodies were raised against two different recombinant *P. falciparum* G6PD-6PDL polypeptides expressed in the vector pGEX (Amersham Biosciences), which contains the glutathione-S-transferase sequence upstream from the polylinker to produce a fusion protein with the insert. Sequences from ntG6PD-6PGL (AAYYICKEIYDKQQINKDGYVVIGLSGGRTPIDVYKNMCLIKDIKIDKSKL) and ctG6PD-6PGL (KILKSIPSIKLEDTIIGQYEKAENFKEDENNDDESKKNHS) (see Figure 1 for their location within the G6PD protein) were amplified and cloned into pGEX. Expression in *E. coli* was achieved following the manufacturer's instructions and the two glutathione-S-transferase/G6PD-6PDL fusion proteins were separately purified using a glutathione sepharose affinity column (Amersham Biosciences). Cleavage of the fusion protein by factor X and subsequent separation in SDS-PAGE provided pure protein for antibody production. Antibodies against ntG6PD-6PGL and ctG6PD-6PGL where raised separately in rabbits but used as a mixture in the Western blot analyses to increase the signal.

FIGURE 1. ***G6PD-6PGL protein and location of antibody reactivity.*** *Scheme showing primary structure of the G6PD-6PGL gene with two main functional domains, the low complexity stretch (black) and the sequences of the peptides corresponding to the two antibodies raised (see text).*

For Western blot analyses, infected red blood cells (IRBCs) from a 10 ml-culture were suspended in 2 volumes of 0.2% saponin in PBS and incubated for 20 min at 37º C, to lyse the RBC membranes. The released parasites were pelleted at 10000 x g for 10 min and washed three times in cold PBS. The parasite pellet was solubilized in 100 µl of TTP (PBS containing 1% Triton X-100 and a protease inhibitor cocktail, Complete Mini, Roche Diagnostics, Mannheim, Germany) and incubated for 30 min at 4º C with frequent vortexing. Next, the soluble parasite fraction was frozen (at –70º C for 5 min) and thawed (at 37º C for 5 min) only once and centrifuged for 10 min at 10000 x g. 10 µg of total protein supernatant was boiled in 5x loading buffer and separated on 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes and probed with the above mentioned antibodies at 1/200 dilutions. Antibody binding was detected by incubation with secondary 1/5000 HRP-conjugated sheep anti-rabbit Ig Abs, followed by chemoluminescence detection using SuperSignal West Pico (Pierce Biotechnology Inc, Rockford, IL). Bands were quantified using Quantity One® software (BioRad, Hercules, CA).

RESULTS

PfG6PD-6PGL protein expression patterns across the intraeryhrocytic cycle

Expression patterns at the protein level were examined through immunodetection of parasite G6PD-6PGL using antibodies against N- and C- terminal separately and a mixture of both of them. Bands with different molecular weight were consistently observed in western blots. As shown in Figure 2, a 107 kDa band corresponding to the theoretical molecular mass of the deduced protein sequence was abundant mainly in mature stages and detected with both antibodies. Also two other abundant bands of 73 and 69 KDa were observed, predominantly in the mature parasite. Thus, the 73 kDa band, detected by the anti-6PGL domain antibody, has a size that includes the N-terminal domain with the expected 6PGL activity and the main low-complexity stretch between both domains. The 69 kDa band, detected by the anti-G6PD domain antibody, match with the

expected size of the G6PD domain. In addition, in the mature stages two other smaller bands were also detected: a 44 kDa band detected with the anti-6PGL domain and a 39 kDa band detected with the anti-G6PD domain, which could result from degradation of their corresponding 73 kDa and 69 kDa band, respectively.

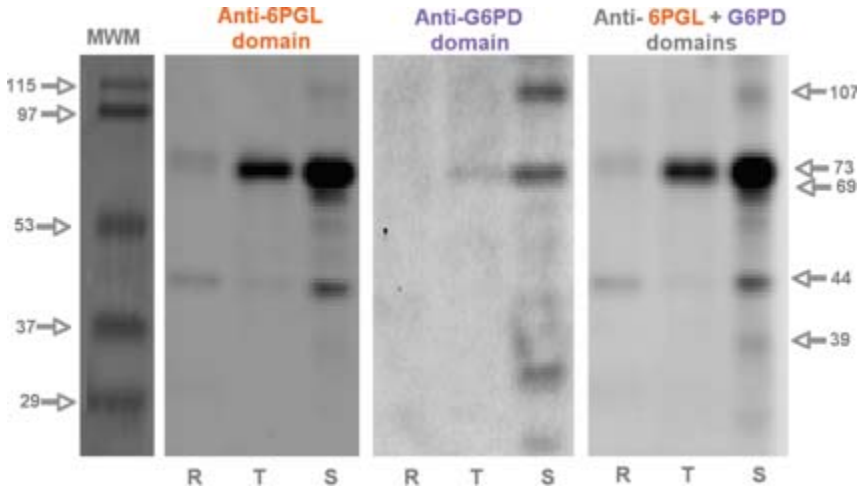

FIGURE 2. ***G6PD-6PGL protein patterns across the intraerythrocytic cycle of P. falciparum.*** *Expression patterns at protein level were examined through immunodetection of the parasite G6PD-6PGL. Parasites harvested at the three main stages, rings (R), trophozoites (T) and Schizonts (S) were transferred and immunodeveloped with three different conditions using anti-6PGL domain, anti-G6PD domain, and a mixture of both of them.*

dsRNA silencing of the PfG6PD-6PGL and expression pattern

In an attempt at silencing the G6PD-6PGL gene, erythrocytes infected with ring-stage *P. falciparum* 3D7 (pyrimethamine-sensitive clone) were electroporated with a dsRNA-G6PD duplex, RNase-free water and dsRNA-Rab5a (the last two as controls) as previously reported (18).

The G6PD-6PGL protein band pattern during the *P. falciparum* intraerythrocyte cycle, as revealed by immunodetection with both antibodies is shown in Figure 3A. In all stages, two main bands of different molecular weight were observed, a 107 kDa band

corresponding to the theoretical molecular mass of the deduced protein sequence and the 73 kDa band with 2 other subforms in the mature parasites.

Compared to the control, gene silencing by dsiRNA reduced G6PD-6PGL immunoreactivity as observed in cultures at 3 and a 24 h (Fig. 3B). Thus, as earlier as 3 h, a 54% and 69% reductions in the 73 kDa and 107 kDa bands, respectively, were observed (Fig. 3C). After 24 h, this effect started to diminish, and only 14% and 19% decreases in the two bands, respectively, were observed (Fig. 3C). These data confirm previous findings that silencing through dsRNA-G6PD instantly took place after 3 h of electroporation at the ring stage, with normal levels gradually restored at the trophozoite stage, 24 h post-transfection (18).

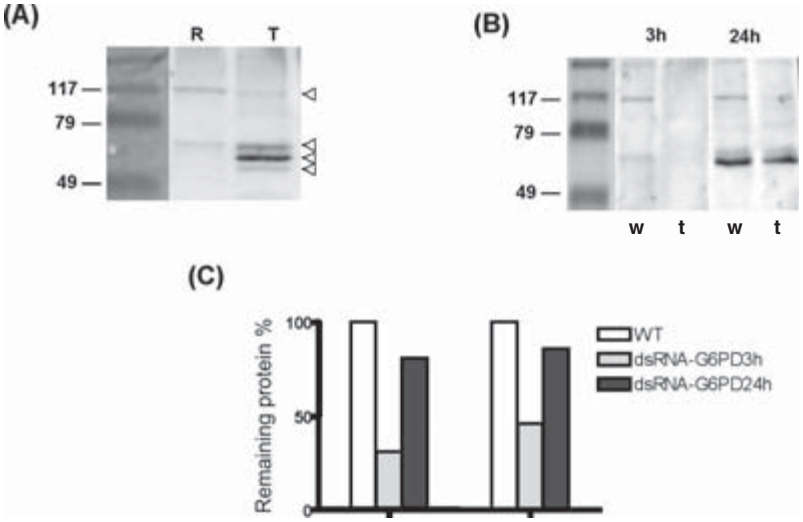

FIGURE 3. ***Effect of dsRNA on parasite protein levels. (A)*** *Immunoblot analysis of wild-type synchronized 3D7 rings and trophozoites. The G6PD-6PGL protein band pattern immunodetected during the intraerythrocyte cycle of P. falciparum includes the 107, 73, 69 and 53 kDa bands labelled with arrows.* ***(B)*** *Immunoblot analysis of synchronized wild-type (w) and dsRNA-G6PD transfected (t) parasites (10 μg of total protein per lane) at 3 and 24 h after electroporation.* ***(C)*** *Immunoblot quantification of synchronized wild-type and dsRNA-G6PD parasites. The 107 and 73 kDa bands signal detected were quantified by densitometry using a Fluor-S MultiImager and Quantity One quantitation software (Bio-Rad). Value for PfG6PD-6PGL in dsRNA-G6PD parasites is expressed as a percentage of control levels in wild-type parasites transfected with water. The positions of molecular mass standards are indicated (kD).*

DISCUSSION

The data presented above indicate that this unique bifunctional *Plasmodium* G6PD-6PGL protein varies in size depending on the parasite developmental stage. Thus, the 69 KDa band detected by the anti-G6PD domain antibody coincides with the expected size of the G6PD domain at the C-terminal, and the 73 kDa band identified by the anti-6PGL domain antibody could have a size that including the essential insertion (24) corresponds to the N-terminal of the protein with the 6PGL activity. Thus, it seems that the bifunctional protein could mature to render two different polypeptides with separate enzyme activities but sharing the essential insertion.

To assess specific biological gene function in the parasite, several systems for the functional analysis of *P. falciparum* genes have been developed, including gene silencing by antisense RNA (14, 18) or more recently, by RNA interference (16-18). Antisense RNA has been found in humans, mice, plants and protozoan parasites such as *P. falciparum*. The fact that endogenous antisense RNAs are widespread in *P. falciparum*, suggests that they could be a natural gene expression regulatory mechanism (28, 29). In our model, *P. falciparum* G6PD-6PGL was silenced *in vivo* through a dsRNA. Although mechanisms of RNAi silencing in many organisms are not well known, this technique has been used to study gene function in a great variety of organisms including other parasites (30). Despite the fact that, so far, the genes encoding the required RNAi machinary have not been detected in any of the currently available *Plasmodium* databases, RNAi silencing has been achieved in *Plasmodium* (16-18). Thus, it could be that the data reported for *Plasmodium*, as well as our results using dsRNA-G6PD, are the consequence of an antisense RNA rather than a direct RNAi effect. However, it is also true that, to date, 60% of the genes predicted for *P. falciparum* have no known homologs, and we have no clues as to their function (4).

The protein expression patterns examined by gene silencing showed that after 3 h of transfection, when most of the parasites are at the ring stage, the complete 107 kDa band predominates in both transfected and not transfected parasites. However, after 24 h of electroporation when the parasites were mainly at the trophozoite stage, the main band was the 73 kDa band. Again, this data suggest

protein size change across parasite life cycle by protein processing or by alternative splicing. To this respect, different sizes of mRNA G6PD-6PGL have been observed in parasites in the ring and trophozoite stages (31). Thus, there seems to be specific *P. falciparum* mechanisms for processing this mRNA, controlled by the parasite's development cycle, which could be unique or shared with other genes (32, 33). The 69 kDa band could coincide with the C-terminal end of the protein corresponding to G6PD activity in such a way that it would not show the N-terminal end that corresponds to the 6PGL activity. Based on the data shown, we can hypothesize a controlled pattern of PfG6PD-6PGL processing during parasite maturation as depicted in Figure 4.

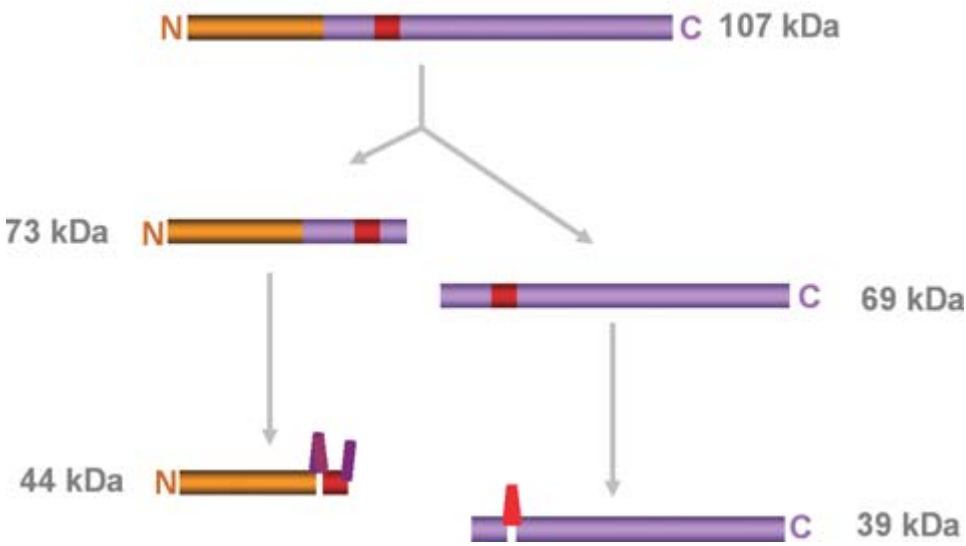

FIGURE 4. ***Hypothetical processing of G6PD-6PGL.*** *The protein product sizes observed by immunodetection analysis across the intraerytrocytic P. falciparum life cycle could be explained by two steps of controlled maturation of the protein to take apart, optimize and recycle, the two functional enzymatic activities in the parasite cell.*

An independent role of the 6PGL function has been addressed, and although its 6-phosphogluconolactone substrate is highly unstable *in vitro*, some increase in the efficiency of the pathway may be evident (21,34). To this respect, the lactonase activity

shown by the bifunctional enzyme is ten times lower than that of the monofunctional domain 6PGL (21), demonstrating its low efficiency for 6-phosphogluconolactone hydrolysis. Moreover, from a structural standpoint, it has also been questioned whether the bifunctional protein is more efficient in producing NADPH than two separate enzymes (21). NADPH is also the co-substrate of *Plasmodium* glutathione reductase and thioredoxin reductase enzymes that protects against oxidative stress caused mainly by digestion of host cell haemoglobin at the late ring-early trophozoite stage (35). Thus, different NADPH efficiencies could be required at different developmental stages with particular specialization by the bifunctional protein (21). Another explanation of this apparent processing, is the genome economy shown by the small *Plasmodium* genome containing a proportionally high number of genes compared to similar genome sizes (36, 37). Several other unique bifunctional enzymes have been described in *Plasmodium* species (38-40) reflecting the parasite's rapid evolution in its constant fight to overcome host defence mechanisms.

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